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Localization of the β-Glucosidases in *Neurospora crassa*

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The β-glucosidases (EC 3.2.1.21) of *Neurospora crassa* were studied with respect to their location in conidia and young mycelia. Aryl-β-glucosidase of conidia was nearly equally divided between extracellular and bound activity. Bound aryl-β-glucosidase was almost all available to substrate. An induction procedure was used to maximize both β-glucosidases in 4 to 6-hr cells. Aryl-β-glucosidase was entirely bound but still mostly (90%) detectable, whereas cellobiase was mostly internal and cryptic. A freeze-thaw cycle or treatment with phenethyl alcohol or deoxycholic acid made the cellobiase detectable without releasing it from the cell. A 10 to 20% increase in cell-bound aryl-β-glucosidase could be obtained by this treatment. Dilute HCl (0.1 N) destroyed the patent aryl-β-glucosidase but not the cryptic aryl-β-glucosidase or the cryptic cellobiase activity in intact cells. This suggested that most aryl-β-glucosidase activity was exterior to the cell membrane but still within the mural space. The thermal stability of patent aryl-β-glucosidase and released cellobiase was found to be higher than in corresponding cell-free extracts. Measurements of K_m suggested a slightly lower affinity for substrate *p*-nitrophenyl-β-D-glucopyranoside by the enzymes in intact cells compared to enzymes in extracts.

We attempted to determine the location of two β-glucosidases (β-glucoside glucohydrolase, EC 3.2.1.21) within the cellular structure of *Neurospora crassa*. The possibility that one of these enzymes, aryl-β-glucosidase, might be located in the space external to the cell membrane was suggested when it was found that this enzyme could be washed from wild-type conidia (5).

Recent studies by Metzenberg (21) showed a mural location for invertase in *Neurospora*. The enzyme was located either in or beneath the cell wall and apparently outside the cell membrane. Mural enzymes were initially identified by their vulnerability to treatment with dilute acid solutions (18) and other inhibitory substances that could not effectively pass the cell membrane (4, 25). These treatments presumably destroyed or inhibited enzymes located outside the cell membrane, while leaving the internal enzymes fully active.

Part of our present investigation paralleled earlier findings with other mural enzymes, and established that aryl-β-glucosidase is primarily a detectable or mural enzyme during any stage of the vegetative life cycle of *Neurospora* in which it is induced. The second β-glucosidase, cellobiase, is predominantly internal and cryptic at all stages, but its activity can be detected in intact-cell assays by specific physical or chemical treatments that alter the cell membrane. These studies on enzyme location also provided a new opportunity for speculation on the true roles of the β-glucosidases in *Neurospora*.

MATERIALS AND METHODS

Chemicals. Cellobiose, deoxycholic acid (DOC), and *p*-nitrophenyl-β-D-glucopyranoside (PNP-G) were products of Calbiochem, Los Angeles, Calif. Phenethyl alcohol (PEA) was obtained from Eastman Organic Chemicals, Rochester, N.Y. Sodium lauryl sulfate was purchased from Nutritional Biochemicals Co., Cleveland, Ohio. Triton X-100 was obtained from Rohm and Haas, Philadelphia, Pa. Tris(hydroxymethyl)aminomethane (Tris) was obtained from Sigma Chemical Co., St. Louis, Mo. Nystatin was the product of E. R. Squibb and Sons, Inc., New York, N.Y.

Media. A modified glycerol-complete agar medium (6) was used for maintenance of *Neurospora* stocks. Mycelia were grown in Vogel's single-strength medium (29) with vitamins and 2% sucrose (w/v) as the carbon source. Induction experiments were carried out in 0.1 M potassium phosphate buffer (pH 6.0) containing 1.0 mM cellobiose as the inducer.

Strains. Wild-type strains of *Neurospora* from several genetic backgrounds were chosen for comparative purposes. Mutant strains designated *gluc-1* (6) contain 10% of wild-type aryl-β-glucosidase activity, and *gluc-2* strains contain less than 1% of wild-type aryl-β-glucosidase activity. *Gluc-2*, a putative allele

of *gluc-1*, was isolated from *gluc-1* and shows no recombination in crosses from which over 200 random spores were tested.

Growth and harvest of conidia. Conidia were grown in Erlenmeyer flasks containing modified glycerol-complete agar medium for 7 days at 25 C (6). To harvest, we added sterile, glass-distilled water to the growth flask and shook the flask gently to suspend the conidia. The conidial suspension was then filtered through glass wool into centrifuge tubes and centrifuged for 5 min at 3,000 × *g* in a Sorvall type SP/X centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.). The conidia were washed twice more with water and centrifuged after each wash. Aseptic techniques were used throughout the growth, harvest, and induction procedures.

Standard induction procedure. Washed conidia were suspended in 10 ml (final volume) of 0.1 N HCl and shaken gently for 5 min. This treatment destroyed all patent β-glucosidase activity without impairing cell viability. The conidia were then centrifuged, and the pellet was suspended in 10 ml of 0.1 M potassium phosphate buffer, *p*H 6.0. Conidia were inoculated into 125-ml Erlenmeyer flasks containing 40 ml of standard induction medium with an inoculum that had an optical density (OD) reading of 0.050 to 0.100 at 600 nm (equivalent to 3×10^6 to 6×10^6 cells per ml of induction medium) in a spectrocolorimeter (model 151; Beckman Instruments, Inc., Spinco Division, Palo Alto, Calif.) in the final suspension. Conidial inocula with an OD reading greater than 0.1 (6×10^6 cells per ml) often resulted in less than maximal activity obtainable when 1.0 mM cellobiose was the inducer.

The inoculated flasks were placed in a reciprocal shaker bath (New Brunswick Scientific Co., New Brunswick, N.J.) set at 25 C and a speed of 160 cycles per min. The flasks were removed from the bath after 5 to 6 hr and chilled in an ice bath. The contents of the flasks were then centrifuged at 3,000 × *g* for 20 min at 5 C. The supernatant fraction was discarded, and the pellet was either refrigerated at 5 C prior to further treatment or frozen at −25 C.

Extraction of conidia or induced cells. Cells were suspended in 15 ml (final volume) of standard buffer and placed in a 50-ml polyethylene beaker with 5 g of chilled, glass, homogenizing beads (no. 16-220; VirTis Co., Gardiner, N.Y.). The suspension was disrupted in a Sonifier (model S-110, Branson Instruments, Inc., Stamford, Conn.) at a setting of 4.3 amp for 1.5 min at 5 C. The disrupted cells were placed in an ice bath at 5 C for 1 hr, and each sample was stirred frequently to maximize enzyme extraction. The suspensions were then centrifuged for 1 hr in a model A "Beta-Fuge" (no. 9RA; Lourdes Instrument Corp., Brooklyn, N.Y.) at a speed of 13,000 × *g* at 5 C. The extracts were frozen for future assay.

Growth, induction, and extraction of mycelia. Erlenmeyer flasks (250 ml) containing 80 ml of growth medium were inoculated with conidial suspensions and placed in the reciprocating incubator-shaker bath at 25 C. After 48 hr, the contents of the flasks were washed twice with sterile distilled water and transferred to similar flasks containing 80 ml of 0.1 M phosphate buffer (*p*H 6.0) plus 1.0 mM cellobiose for 6 hr. The mycelial pads were then harvested by filtration on Whatman no. 1 paper, washed twice with water, and frozen at −25 C.

For extraction, the mycelial pads were removed from the freezer and placed in 30 ml of 0.01 M potassium phosphate buffer (*p*H 6.0) and 10 g of glass homogenizing beads in the 50-ml chamber of Omni-Mixer no. 115 (Ivan Sorvall, Inc.) for 10 min at a setting of 8. The resulting slurry was then treated with a Branson Sonifier for 1 min at 5 C at a setting of 6.3 amp and allowed to extract for 1 hr at 5 C with frequent stirring prior to centrifugation for 1 hr at 13,000 × *g* at 5 C.

Assay methods. β-Glucosidase activity in cell-free extracts was assayed by using a discontinuous method (5) with PNP-G as substrate.

β-Glucosidase activity in intact cells and modified cells was also assayed by the discontinuous PNP-G method with the following modifications. Each cell sample was evenly suspended, and a small sample was removed for a cell count (hemocytometer). Cell samples of 0.1 ml were pipetted into test tubes (10 by 75 mm); 0.9 ml of standard buffer containing 1 mg of PNP-G was added to each tube to start the reaction. This mixture was shaken gently for 10 min, and the enzyme reaction was stopped by the addition of 0.5 ml of 1 M Tris. Cells were removed by centrifugation at 3,000 × *g* for 10 min. This assay procedure was also followed for broken-cell preparations prepared by treatment with a Branson Sonifier and assayed as suspensions.

The ratio of aryl-β-glucosidase to cellobiase in all samples was determined by comparing two samples, one of which had been heated for 1 min at 60 C. Cellobiase activity was destroyed by this treatment, and the activity remaining after heat treatment was attributed to aryl-β-glucosidase. The heated samples were immediately cooled in an ice bath for 15 min and then returned to 25 C for assay.

Calculations of activity. One enzyme unit is defined as 1 μmole of PNP released per min at 25 C as measured at 410 nm in a Beckman 151 spectrocolorimeter. Specific cell activity is expressed as units per 10^8 cells.

RESULTS

Distribution of β-glucosidase activity in conidia and 6-hr cells. Previous experiments showed that significant amounts of aryl-β-glucosidase could be removed from *Neurospora* conidia by washing with water (5). These washed conidia still contained activity that could be detected after disruption of the cells. In the experiments reported here, we have found that the activity which remained with the cells after washing could also be measured directly in assays by using undisrupted conidia, indicating that the enzyme activity might be located within or under the cell wall space.

In the initial experiments a variety of wild-type strains were examined both for soluble and cell-

bound β-glucosidase activity. The experiment shown in Table 1 confirmed our original observation that a substantial portion of the enzyme activity was indeed solubilized by simple washing with water. By contrast, a larger portion of β-glucosidase activity still remained with the conidia and was easily detectable by using PNP-G as substrate with intact cells. The ratio of external to cell-bound enzyme was characteristic of each strain.

The distribution of β-glucosidase activities from conidia of two types of mutant strains was also examined in the experiment shown in Table 1. The mutants had been characterized originally by their low levels of aryl-β-glucosidase activity, yet their cellobiase levels were normal (15). The ratio, however, of cell-bound to free enzyme activity was much lower than that in the wild-type preparations. These differences of distribution are difficult to explain since the aryl-β-glucosidases obtained from *gluc-1* and *gluc-1*$^+$ were indistinguishable in all properties studied (15).

Acid treatment and induction. In parallel with work on aryl-β-glucosidase, we also tried to determine the location of cellobiase in cells from a variety of strains. Cellobiase activity was, however, generally at low levels in intact conidia or mycelia or in extracts of these cells. The detectable cellobiase activity was often so small that it was impossible to determine with certainty. An investigation of the location of cellobiase would therefore require cells with this enzyme activity at levels high enough to follow with accuracy. This led to the development of a procedure using cellobiose as inducer to increase the levels of both enzymes. In this way, the distribution of both β-glucosidases was studied under conditions in which their activities were easily determined. Other details of general induction of both β-glucosidases will be reported elsewhere.

A part of the procedure that should be mentioned here relates to the treatment of conidia with 0.1 N HCl for 5 min. This eliminated all patent β-glucosidase activity. Prior to the induction, however, acid-treated cells possessed a residue of cryptic activity (less than 10% of the of total) that could be released by sonic disruption. We have used acid-treated cells in our standard induction experiments because: (i) their viability was not altered by the treatment; (ii) the patent activity was eliminated so that one could determine the new enzyme activity produced during the induction; (iii) cellobiase activity was usually greater in induced cells as a result of initial acid treatment. We assume that the acid treatment eliminated aryl-β-glucosidase which could destroy the β-glucosidase inducer entering the cell.

Liberation of enzyme activity. Conidia prepared in the standard way were induced and examined for both patent and cryptic β-glucosidase activity (Table 2). Both enzymes were patent in intact cells with aryl-β-glucosidase clearly the main activity. Samples of these cells were disrupted by treatment in a Branson Sonifier, producing a mixture of broken cells and liberated cell con-

TABLE 1. *β-Glucosidase activity in conidia and water of extraction*

Strain	Genotype	β-Glucosidase activity[a]	
		Extract	Intact conidia
74-OR8-1a	*gluc*$^+$	22.5	40.8
533-A	*gluc*$^+$	19.2	25.1
CM-168	*gluc*$^+$	20.0	38.1
354-A	*gluc*$^+$	21.1	27.5
STA-4	*gluc*$^+$	28.6	37.4
74-OR23-1A	*gluc*$^+$	27.8	37.4
RB-1(19)	*gluc*$^+$	25.6	37.4
RB-1(30)	*gluc*$^+$	17.1	29.7
27-(2-5)	*gluc-1*	3.3	1.6
33-(3-7)	*gluc-1*	3.6	1.5
CM62(2-2)a	*gluc-2*	0.3	0.3
CM75(9-5)a	*gluc-2*	0.3	0.3

[a] Conidia were harvested in a dry condition by shaking growth flasks over a dry surface, suspended, and washed three times in distilled water (10 ml per wash). The wash volumes were pooled for standard β-glucosidase assay. Activity is expressed as units per 10^8 cells.

TABLE 2. *Liberation of β-glucosidases from induced cells*[a]

Strain	Enzyme activity[b]			
	Cellobiase		Aryl-β-glucosidase	
	Intact cell	Sonified cells plus extract[c]	Intact cell	Sonified cells plus extract
STA-4	3.8	19.3	25.0	25.8
74-OR8-1a	4.4	12.4	15.8	16.2
74-OR23-1A	2.1	13.2	19.3	24.3
RB1(19)	5.6	14.0	26.3	29.2

[a] Induction procedure was standard and includes washing the conidia with 0.1 N HCl to remove patent aryl-β-glucosidase. Intact cells were assayed immediately after induction. All strains were wild type.

[b] All activity is expressed as units per 10^8 cells.

[c] The mixture of sonically disrupted cells and extract was assayed by the intact-cell method.

tents. This mixture had higher levels of cellobiase, whereas aryl-β-glucosidase activity increased only slightly.

In another experiment (Table 3), the broken-cell fragments were separated by centrifugation and resuspended for assay in the same volume of standard buffer. Much cellobiase activity was released by sonification, whereas proportionally more of the aryl-β-glucosidase remained bound to the cell. These two experiments showed that aryl-β-glucosidase was mostly patent in intact cells, whereas cellobiase was highly cryptic prior to disruption of the cells. This information is compatible with the mural location of aryl-β-glucosidase and the intracellular location of cellobiase. Much of the aryl-β-glucosidase stays bound to fragments after treatment of cells in a Branson Sonifier.

Alteration in patency of cellobiase in whole cells. The crypticity of cellobiase in intact cells might be explained by the interposition of the cell membrane between the exogenous substrate and the endogenous enzyme. The disruption of the cell membrane should therefore make cellobiase activity more detectable in whole-cell preparations. To simulate this condition in another way, we used several reagents that were shown to alter the integrity of the membrane in *Neurospora* and other fungi (3, 12, 13). In addition, we applied a freeze-thaw cycle already shown to disrupt membranes by a different mechanism (20, 21).

Whole induced cells of a wild-type strain were exposed to selected surface active agents and assayed after 30 min (Table 4). Treatment with PEA, DOC, sodium lauryl sulfate (SLS), Triton X-100, and nystatin significantly increased the patency of cellobiase activity. The patency of aryl-β-glucosidase was increased only slightly or, in the case of nystatin, decreased. None of the reagents released β-glucosidase activity of either type from the cell into the surrounding medium.

An experiment showing the effects of increasing concentrations of PEA or DOC, plus a freeze-thaw cycle, on induced intact cells is illustrated in Table 5. With freeze treatment standard in each case, concentrations of PEA up to 50 mM stimulated an increase in cellobiase activity, but concentrations of PEA greater than 5 mM resulted in decreased aryl-β-glucosidase activity. This inhibitory effect of PEA on aryl-β-glucosidase activity was probably a specific competitive inhibition (*unpublished data*). DOC also increased cellobiase activity, but the effect was noticeably less at similar concentrations. The higher DOC concentrations were apparently also inhibitory to cellobiase. The order of treatment seemed to affect the amount of enzyme liberated, since more activity was obtained by adding the 1.0 mM PEA first and then freezing. With 1.0 mM DOC, the reverse was true.

In a separate set of experiments, 1.0 mM PEA was incubated with partially purified solutions of cellobiase and aryl-β-glucosidase. In all of the experiments (including freezing, heating at 60 C, and others), no stimulation or inhibition of either of the enzymes by PEA was noted.

Inactivation of cell-bound β-glucosidase activity by acid. The following experiment was designed to determine the effect of acid treatment on detectable and cryptic β-glucosidase activities in time. Conidia were induced by the standard method, including the preinduction acid treatment, to remove initial detectable β-glucosidase. After the 5-hr induction period, these cells were exposed again to 0.1 N HCl, and samples were removed at various intervals during the 5 min. They were immediately neutralized in standard buffer, and one portion of each of these samples

TABLE 3. *Distribution of β-glucosidases in induced cells*

Strain	Enzyme activity[a]					
	Cellobiase			Aryl-β-glucosidase		
	Intact cells[b]	Cell-free extract	Broken cells[c]	Intact cells	Cell-free extract	Broken cells
74-0R8-1a	1.1	4.4	1.6	8.8	4.2	5.0
74-0R8-1a	2.0	5.2	1.6	9.3	4.7	5.1
74-0R23-1A	0.8	6.5	0.9	7.8	4.2	3.6
74-0R23-1A	2.3	5.4	2.4	9.7	4.7	4.8

[a] Enzyme activity is expressed as units per 10^8 cells.
[b] Intact cells were assayed immediately after induction.
[c] Broken-cell preparation consisted of the cells treated in a Bronson Sonifier (centrifuged and resuspended) and assayed as intact cells.

TABLE 4. *Effect of surface active agents on cell-bound β-glucosidases*[a]

Reagent[b]	Enzyme activity[c]	
	Cellobiase	Aryl-β-glucosidase
None	3.7	17.3
PEA	8.6	17.3
DOC	6.3	18.3
SLS	9.7	24.6
Triton X-100	25.1	22.4
Nystatin	29.3	11.0

[a] Wild-type strain 74-OR23-1A was used throughout. Induced cells were incubated for 30 min at 25 C with the reagents listed, and then assayed by using standard intact-cell assay.

[b] Abbreviations: PEA, phenethyl alcohol in absolute ethyl alcohol; DOC, deoxycholic acid; SLS, sodium lauryl sulfate. All concentrations were 1.0 mM, except Triton X-100 (0.25%) and nystatin (50 μg/ml). Nystatin was prepared in absolute methanol.

[c] Enzyme activity is expressed as units per 10^8 cells.

TABLE 5. *Release of β-glucosidases into the mural space by treatment with PEA, DOC, and freezing*[a]

Treatment	Reagent concentration	Enzyme activity[b]			
		Cellobiase		Aryl-β-glucosidase	
		PEA	DOC	PEA	DOC
	mM				
None		2.5	2.5	56.5	56.5
Freeze only[c]		19.3	19.3	71.7	71.7
Reagent only	1.0	16.7	4.3	47.3	54.7
Reagent, freeze[d]	0.1	20.9	43.5	78.2	65.1
Reagent, freeze[d]	0.5	14.0	35.9	78.3	51.5
Reagent, freeze[d]	1.0	58.1	38.2	80.2	59.6
Reagent, freeze[d]	5.0	65.5	16.9	76.9	75.5
Reagent, freeze[d]	10.0	73.9	23.1	73.9	72.7
Reagent, freeze[d]	50.0	87.9	26.6	51.7	56.7
Freeze, reagent[e]	1.0	38.2	48.3	59.6	69.3

[a] Standard induction procedure and intact-cell assay were used with wild-type strain 74-OR8-1a.

[b] Enzyme activity is expressed as units per 10^8 cells.

[c] Cells were brought quickly to −25 C and left for 18 hr, and then allowed to thaw at 25 C.

[d] The reagents were allowed to incubate with the cells at 25 C for 30 min before freezing.

[e] In this sample, the cells were frozen at −25 C first, and the reagent was added to the cells during the thaw. The cells plus reagent were incubated for 30 min at 25 C and then assayed.

was assayed for whole-cell activity. A similar portion of each sample was incubated with 1.0 mM PEA, frozen for 12 hr at −25 C, and then assayed to determine the effect of acid treatment on cryptic activity of both enzymes.

The detectable levels of induced aryl-β-glucosidase were high prior to exposure to acid (zero time, Fig. 1A), and a typically low level of patent cellobiase activity was noted in these cells. The aryl-β-glucosidase activity decayed rapidly in the first 15 sec of exposure to acid, whereas the cellobiase activity showed little if any decline during the same interval. These patent enzyme activities were not completely eliminated by a 5-min acid treatment in these 5-hr induced cells, as they were in conidia. One possible reason for this difference was the high cryptic level of both enzymes in induced cells at the time of exposure to the acid compared to the relatively low cryptic β-glucosidase activity in conidia (Table 2). What appeared to be an acid-resistant residue of β-glucosidase activity in induced cells may be activity released from crypticity during the interval between acid treatment and assay.

The levels of cryptic β-glucosidase activity that remained after acid treatment of the induced cells were assayed after an exposure to PEA and freezing (Fig. 1B). The cellobiase activity released by this treatment was initially eight times greater than the cellobiase of the control cells. Exposure to acid reduced cryptic activity by about 33% during the 5-min interval. The gradual nature of the decline in activity suggests that cellobiase was protected in some way from acid inactivation, presumably by the same barrier mechanism that made it unavailable to its substrate.

During acid treatment, aryl-β-glucosidase

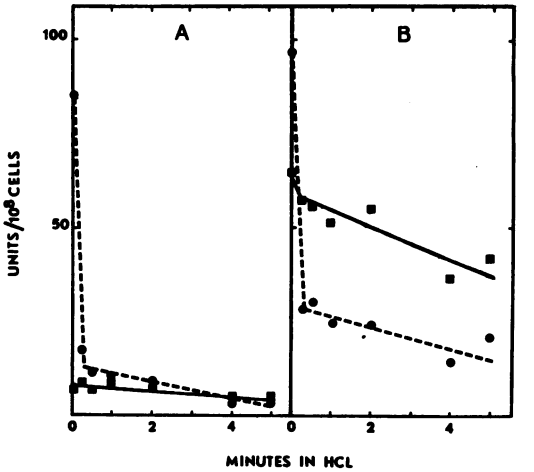

FIG. 1. *Inactivation of postinduction cell-bound β-glucosidase activity by 0.1 N HCl. (A) Detectable activity in unmodified intact cells; (B) total activity in cells after treatment with PEA and a freeze-thaw cycle. Wild-type strain 74-OR8-1a was used. Induction included initial HCl treatment, and assays were by standard intact-cell method. Symbols: ●, aryl-β-glucosidase; ■, cellobiase.*

activity declined sharply in the first 15 sec, and then declined gradually for 5 min (Fig. 1B). We assume that the aryl-β-glucosidase that was destroyed at a slower rate during the exposure to acid was internal. It seems that cells induced for 5 hr did not release all of the aryl-β-glucosidase into the mural space. Subsequent experiments suggest that this cryptic activity eventually becomes patent in time. The ratios of the two β-glucosidase activities in extracts obtained from broken cells were very close to those shown in cells exposed to PEA-freezing.

Comparative thermal properties of β-glucosidases in conidial washes, whole cells, and cell-free extracts. The thermal properties of the β-glucosidases were used in our laboratory as the standard method of distinguishing between these enzymes when they are both present in solutions. The following experiments demonstrate differences in the thermal properties of both β-glucosidases free of, or while still bound to the cell. Thermal properties of aryl-β-glucosidase are shown in Table 6. The half-life at 60 C was determined for aryl-β-glucosidase in unmodified cells induced for 6 hr as well as three types of cell-free preparations. Five wild-type strains were the sources of enzyme activity. These values were obtained from experiments in which the preparations were heated for 30 min, and samples were taken and quickly cooled at 3-min intervals.

Both conidial wash preparations and intact-cell preparations showed a logarithmic rate of decay, suggesting one enzymatic component, aryl-β-glucosidase. Extracts from induced cells showed a sharp initial drop in the first minute, and then a logarithmic rate of decay for the rest of the interval, suggesting at least two enzymes. This initial decline in induced cells was presumed to be due to decay of cellobiase activity, which was essentially destroyed by exposure to 60 C for 2 min (6). There was an expected variation in the thermal stability of aryl-β-glucosidase among the wild-type strains used, but the range was not as extreme as the difference previously noted for certain exotic strains (16). There was, however, a significant and consistent difference in the half-life of aryl-β-glucosidase in conidial washes compared to intact-cell activities. A similar but smaller difference was shown between extracts and intact cells. In general, the aryl-β-glucosidase activity of intact cells was consistently more stable than in either of the other two preparations. The basis for these differences in stability of aryl-β-glucosidase in these four preparations is still being investigated.

To obtain a valid inactivation rate for cellobiase in similar preparations, two additional experimental conditions were used. First, a lower temperature (46 C) was selected to allow a rate of enzyme inactivation that could be measured with sufficient accuracy in 30 min. Second, *gluc-2* strains were used as the source of cellobiase activity, since in these strains cellobiase inactivation was determined without a masking effect from aryl-β-glucosidase.

The data in Table 7 show the thermal properties of the cellobiase from several *gluc-2* strains. The cellobiase was made detectable by the standard freeze-thaw cycle with PEA, but the activity was still associated with the cell. As in the case of aryl-β-glucosidase, cellobiase was more stable in intact-cell preparations than in extracts. The enzyme activity in extracts of 48-hr mycelia showed

TABLE 6. *Thermal half-life at 60 C of aryl-β-glucosidase from different preparations of wild-type strains*[a]

Strain	Source of aryl-β-glucosidase[b]			
	Conidial wash	Cell extract (6 hr)	Mycelial extract (48 hr)	Intact cells (6 hr)
74-OR8-1a	17	61	40	79
STA-4	18	45	56	99
74-OR23-1A	24	47	57	105
CM158(4)a	21	53	67	86
RB-1(19)	15	61	49	173

[a] The half-lives at 60 C expressed in min were calculated from seven samples taken during a period of 30 min for conidial wash and cellular extract and 60 min for intact cells.

[b] Intact-cell preparations and extracts were prepared by standard methods. The intact cells were not frozen or incubated with PEA before assay.

TABLE 7. *Thermal half-life at 46 C for cellobiase from different preparations of gluc-2 strains*[a]

Strain	Source of cellobiase[b]		
	Cell extract (6 hr)	Mycelial extract (48 hr)	Intact cells (6 hr)
CM62(2-2)a	15	21	30
CM75(9-5)a	10	18	25
RB-1(3)a	14	18	35
RB-1(83)a	13	19	50
RB-1(93)A	9	14	29

[a] The half-lives expressed in min were determined by removing seven samples from a bath at 46 C over a period of 30 min.

[b] Whole-cell preparations and extracts were prepared by standard procedures; PEA and freezing methods were used with the intact-cell preparations. Hours indicate time from inoculation.

a slightly greater stability than the activity of the extracts of conidia induced for 6 hr.

Comparative K_m values of β-glucosidases in conidial washes, whole cells, and cell-free extracts. The apparent substrate affinities (Michaelis constants) of aryl-β-glucosidase and cellobiase were determined by using intact cells and cell extracts. The aryl-β-glucosidase K_m values were established by using intact conidia that had been induced for 6 hr. Cell-free extracts were also prepared from these 6-hr cells as well as from 48-hr mycelia.

The results in Table 8 show that there was little difference in the substrate affinity for PNP-G among the wild-type strains used in the three types of preparations. However, extracts of conidia induced for 6 hr had aryl-β-glucosidase K_m values consistently lower than those of the other two preparations in all strains. Intact-cell preparations in three of the strains had the highest K_m values of the three preparations, suggesting that the mural aryl-β-glucosidase in these strains might not be as accessible to the PNP-G as this same enzyme would be in a cell-free extract.

A similar experiment to determine the K_m values of cellobiase with PNP-G as substrate is shown in Table 9. The whole cells of *gluc-2* strains were modified by a freeze-thaw cycle and PEA treatment prior to assay to increase the ability to detect cellobiase. The results indicate that cellobiase under these conditions may be less accessible (higher K_m) when associated with the cell than in a cell-free extract. The differences are relatively small, but are in the same direction as those shown for K_m values of aryl-β-glucosidase in Table 8. Again this may be evidence that the cell wall may create a partial barrier between PNP-G and the cellobiase made patent by PEA and freeze-thaw treatment.

In both Tables 8 and 9, the variation in K_m values between strains is sometimes less than the variation seen between the three types of enzyme preparations. Caution should be shown in attributing a K_m value to either of the β-glucosidases without carefully noting the origins of the preparations used.

TABLE 8. *Michaelis constants of aryl-β-glucosidase from wild-type strains*

Strain	K_m values		
	Conidial extract (6 hr)	Mycelial[a] extract (48 hr)	Intact[b] cell (6 hr)
	mM	mM	mM
74-OR8-1a	0.69	1.50	2.70
STA-4	0.75	1.60	2.50
74-OR23-1A	0.52	1.40	1.40
CM158(4)A	0.67	1.70	1.50
RB-1(19)	0.38	1.40	1.80

[a] Both extracts were prepared and assayed by standard methods. Extract samples were heated for 2 min at 60 C to eliminate cellobiase activity from the assays. PNP-G was used as the substrate.

[b] The intact cells were induced and assayed under standard conditions, but to insure exclusion of cellobiase activity from the assays, the cells were not frozen prior to assay.

TABLE 9. *Michaelis constants of cellobiase from several gluc-2 strains*

Strain	K_m values		
	Conidial extract (6 hr)	Mycelial[a] extract (48 hr)	Intact[b] cells (6 hr)
	mM	mM	mM
CM62(2-2)a	0.72	0.65	0.89
CM75(9-5)a	0.40	0.44	0.90
RB-1(3)a	0.75	0.36	0.94
RB-1(83)a		0.69	0.96
RB-1(93)A	0.40	0.59	0.79

[a] Extracts were prepared and assayed by standard methods. PNP-G was used as the substrate.

[b] The intact cells were grown, induced, and assayed as described in Materials and Methods. Cells were frozen at −25 C and thawed twice before they were assayed.

DISCUSSION

The discovery of the patency of aryl-β-glucosidase and the crypticity of cellobiase in intact-cell preparations helps to clarify the individual functions of these enzymes in β-glucoside metabolism. These properties suggest separate locations that may account for some of the differences in physical properties that have been observed with in vitro studies in our laboratory (Table 10). Despite these differences, both enzymes attack a wide range of β-glucosides, and there is a high degree of overlap in substrate specificity (2, 5, 6, 14, 15). The overlapping specificities seem to contradict the concept that cell economy would best be served by enzymes that have separate functions and separate regulatory mechanisms. There is, however, a distinct difference in the efficiency with which each β-glucosidase attacks cellobiase and PNP-G. The common names "aryl-β-glucosidase" and "cellobiase" only indicate which type of substrate is attacked most effectively by each enzyme. When we consider the differences in the physical properties of the two β-glucosidases in extracts, and especially observe that they

TABLE 10. *Properties of the β-glucosidases of Neurospora*

Property	Aryl-β-glucosidase	Cellobiase
*p*H optimum	5.0	6.1
K_m (PNP-G)	1.5 mM	0.55 mM
K_m (cellobiose)	6.1 mM	0.25 mM[a]
Thermal half-life, 60 C	14 to 50 min[b]	0.5 min
Thermal half-life, 46 C	10 hr	17 to 50 min
Ammonium sulfate ppt	65 to 70% saturated	55 to 60% saturated
Main transglucosylase product from cellobiose	Gentiobiose	Laminaribiose
Elution from diethylaminoethyl, cellulose	0.12 M KCl	0.01 M KCl
Inducer (most active)	Cellobiose	Cellobiose
Membrane electrophoresis (mm/hr at 200 v towards anode)	32	28
Primary location in cell	Mural	Internal
Molecular *weight* (approx)	168,000	80,000

[a] All K_m values represent an average of several determinations.

[b] Aryl-β-glucosidase activity in conidial washes has a much lower half-life than in extracts of broken cells. In all cases, the value obtained depends on the strain and the type of extract or intact-cell preparations used.

are located in different parts of the cell, it seems that their functions in the cell are indeed distinct.

At first glance, the presence of these two β-glucosidases that are easily separated by electrophoresis suggests the phenomenon of isozymes in the multimolecular forms found in animals and higher plants (19, 26). This dual activity is also reminiscent of the heavy and light forms of invertase (27) or possibly the two β-galactosidases (1) found in *Neurospora*. The β-glucosidases in *Neurospora* are probably not multimolecular forms of the same peptide, since each is controlled by separate genes (6, 24); nor can the β-glucosidases be subdivided or hybridized by using any of the standard reagents such as urea or guanidine HCl (22).

Aryl-β-glucosidase is apparently a mural enzyme in the same sense as the invertase of *Neurospora* (21). The immediate patency of the mural enzymes in intact-cell preparations readily distinguishes this class from those cryptic enzymes, such as cellobiase, that become detectable only after treatment of the cell with PEA or similar reagents. These cryptic enzymes are not quickly destroyed by dilute acid treatment of the cell, nor are they bound to the cell wall fraction after disruption of the cell. There is, however, another class of enzymes bound to the cell wall but only slightly patent in intact conidia without additional treatment. For example, the laminarinase activity of intact conidia can be increased 10-fold by a treatment with butanol (17). By contrast, aryl-β-glucosidase activity is only increased 10 to 20% by similar surface active agents. This could mean that there are two types of mural enzymes distinguished by this difference in initial patency. This difference may be more apparent than real, since the chain length of the substrate laminarin may be partly responsible for the low apparent patency of laminarinase. Butanol may alter the cell wall to allow access to the substrate. By contrast, the substrate PNP-G, a much smaller molecule, may find access to aryl-β-glucosidase by the normal mural channels without the changes caused by butanol. By this theory, the small increase in aryl-β-glucosidase that we find after solvent treatment could result from an alteration of the cell wall elements rather than the cell membrane. Yet we have found that the cryptic portion of aryl-β-glucosidase in intact cells is as resistant to dilute acid as is cellobiase. The cryptic aryl-β-glucosidase, as well as cellobiase, must therefore be shielded by the same structure, the cell membrane.

As yet there are few general answers to the following problems related to mural enzymes. (i) How do these enzymes escape the cell membrane? (ii) To what extent are they bound to the exterior of the cell membrane? (iii) Is their condition in the mural space bound or free? That is, are they bound to a cell wall component such as chitin, or are they only trapped between the cell wall and the membrane? Some evidence for the condition of enzymes in the mural space is as follows. The removal of the cell wall in both yeast and *Neurospora* allows the free excretion of invertase (10, 27). These mural enzymes, at least, are not bound to the membrane under these conditions. There is some indirect evidence for the covalent bonding of certain mural enzymes to the cell wall in *Neurospora*. We found that well over half of the aryl-β-glucosidase cannot be re-

moved from cell wall debris by treatment in a Branson Sonifier; the enzyme could, however, be trapped rather than bound (28). A major part of laminarinase and trehalase is also bound to the cell wall fraction (9, 17). More conclusively, D. Braymer has observed that there is a residue of invertase that is removed from the wall with chitinase treatment (*personal communication*). Since hexosamine is bound to invertase (21), this amino sugar could be the bridge through which a portion of the enzyme is attached to the wall. The presence of hexosamine also may mean that the amino sugar plays a part in a general excretion mechanism that has been postulated for glycoproteins (7).

We investigated the possibility that the cell wall or the environment created by the cell wall may alter the properties of the mural enzymes. The thermal inactivation properties of aryl-β-glucosidase were compared in situ and in extracts, and our results suggest that this enzyme is more stable when associated with the cell. To explain this phenomenon, we postulate that the cell wall or other localized components in that space tend to favor a more stable configuration for β-glucosidases by either hydrogen bonding, covalent linkage, or by other less direct effects. Our results, however, contrast with studies in other laboratories in which mural enzymes exhibit thermal properties that are the same in situ and in extracts (11, 21).

Cellobiase made detectable by PEA treatment and a freeze-thaw cycle is also more stable than the cellobiase of extracts.

The substrate affinity of aryl-β-glucosidase for PNP-G tends to differ slightly in intact cell preparations and in extracts. The K_m values for extracts are often lower, suggesting either a greater accessibility of the substrate to the active site of the enzyme or a change of enzyme configuration when it is released from the mural space. These results are not found with the invertase of *Neurospora* (22) or the β-glucosidases of yeast (11), but are noted in the β-galactosidases of *Escherichia coli* (11). It seems that, at least in the case of aryl-β-glucosidase, some of the main physical properties of this enzyme differ in the bound and the free states. The cell wall location may therefore be significant in modifying the function of the β-glucosidases and perhaps other mural enzymes. This situation seems analogous to the different behavior that mitochondrial malate dehydrogenase shows when bound or free (23).

Cellular location of enzymes is apparently not determined by molecular size alone, since aryl-β-glucosidase molecular weight) (168,000, is mural, whereas cellobiase (80,000, molecular weight) is essentially intracellular. Some other factor such as charge or addition of carbohydrates or lipid moieties may be involved in the differential excretion of these enzymes (7). Mural enzymes may indeed have to be above a critical minimum size to avoid passing the cell wall and becoming truly free (28). To explain the free aryl-β-glucosidase and other extracellular carbohydrases (5) found in conidial preparations, further studies will have to examine the mechanisms underlying the formation of the conidial wall layers as they are synthesized at the hyphal tip (8).

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